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Award Number: DAMD17-03-1-0525

TITLE: Chemokine Blockade in Combination with Cytoreductive Conditioning in Metastatic Breast Cancer

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REPORT DATE: August 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                          Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                            Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 01-08-2006	2. REPORT TYPE Final	3. DATES COVERED *(From - To)* 7 Jul 2003 – 6 Jul 2006

4. TITLE AND SUBTITLE

Chemokine Blockade in Combination with Cytoreductive Conditioning in Metastatic Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0525

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Nevada
Reno, Nevada 89557

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Chemokines have been shown to provide angiogenic effects. Blocking chemokines can reduce metastatic breast cancer cell growth. We proposed that cytoreductive conditioning will force the tumor to rely on angiogenesis as a survival mechanism and thus make the tumor more susceptible to chemokine blockade. We demonstrate that tumor cells increase production of IL-8 and MCP-1 transcripts in vivo in response to IL-8 neutralization and/or gamma irradiation and cytoxan administration. We have also demonstrated that IL-8 but not MCP-1 can function as autocrine growth or survival factor for MDA-231 cells in vitro but neutralization requires high concentrations of antibody. This observation is consistent with the more significant anti-tumor effect observed with anti-IL-8 as a single agent and suggest that IL-8 promotes tumor cell growth through multiple mechanisms. We also showed that administration of anti-IL-8 and anti-MCP-1 can inhibit the formation of human breast cancer lung metastasis in a xenograft model.

15. SUBJECT TERMS
Angiogenesis, chemokines, experimental therapies, MCP-1 interleukin-8

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	12	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION:

Chemokines are thought to play a diverse role in tumor progression. They can promote angiogenesis and also promote metastatic spread of a variety of tumors. We have previously demonstrated that chemokine blockade using antibodies to pro-angiogeneic chemokines such as MCP-1 can produce anti-tumor effects against metastatic human breast carcinoma cells in vivo. However, many human breast carcinomas can produce multiple chemokines and the effects of "stress" in this response as a result of cytoreductive conditioning are not clear. It was predicted that chemokine therapy as a single agent modality will achieve only limited efficacy due to redundancy of chemokines produced by the tumor. It was also predicted that "stressing" the tumor with cytoreductive conditioning will make the tumor more reliant on establishing angiogenic pathways and thus more susceptible to chemokine blockade. Prior to this project, chemokine blockade has only been applied as a single agent. It is possible that if the tumor is placed under a "stress" condition, such that occurs after conventional chemotherapy, that greater reliance of angiogeneic pathways may make the residual tumor more susceptible to chemokine blockade. To test this hypothesis 3 specific aims were devised. The first specific aim assessed the effects of cytoreductive conditioning on chemokine production using both in vitro and in vivo. The second specific aim targeted two chemokines that we had shown to individually play a role in human breast cancer cell progression, IL8 and MCP-1, on breast cancer cell survival in vivo. The third specific aim used chemokine blockade in a xenograft model of human metastatic breast cancer cell progression after cytoreductive conditioning. Inhibition of angiogenesis has shown great promise in the treatment of cancer but it has become increasingly clear that use of it as a single agent modality will achieve only limited success, particularly in instances of advance metastatic disease. This proposal attempted to directly generate translational results to optimize the application of chemokine blockade in cancer.

BODY:

The planned tasks, as detailed in the statement of work, were three-fold. (1) To determine the effects of "stress" on human BC cells ability to produce pro-angiogenic chemokines. To determine the effects of cytoreductive conditioning (i.e. chemotherapy or irradiation) on chemokine production by human BC lines placed in immunodeficient SCID mice. (2) To determine the efficacy of chemokine blockade on human BC growth/progression both in vitro and in vivo. Determination of anti-tumor efficacy using both primary and metastatic tumor models. Assessment of anti-angiogenic effects of using blockade of both IL8 and MCP-1 pathways. And (3) to determine the effects of chemokine blockade in conjunction with chemotherapy/irradiation.

Task 1a. To determine the effects of "stress" on human BC cells ability to produce pro-angiogenic chemokines. To assess the effect of "stress" on the ability of human breast cancer cells to produce pro-angiogenic chemokines we have initially focused on the cell line MDA-231. We have explored three forms of "stress"; gamma irradiation, serum starvation and hypoxia. We have observed that the breast cancer cell line MDA-231 can undergo cell cycle arrest and growth inhibition in culture without loss of cell viability up to 72 hours post ionizing irradiation (data not shown). This growth arrest is accompanied by increased production of the pro-angiogenic chemokines MCP-1 and IL-8. The irradiation dose effect on increased chemokine production is more striking when calculated by the cells present at the completion of the culture period (figure

1A and B). Normalization of the results accounts for the growth arrest associated with the treatment. Gamma irradiation induces a 1.6 fold increase in MCP-1 and a 2.1 fold increase in IL-8 following exposure to 300 cGy and a 2.7 fold increase in MCP-1 and 6.8 fold increase in IL-8 after 2000 cGy on a per cell basis. Surprisingly, the induction of IL-8 is a rather late event as evidenced by mRNA analysis at 24, 48 and 72 hours post-irradiation of MDA-231 cells (figure 2), where the maximal difference in transcription is observed at the later time point. Other chemokines that were assessed in MDA-231 include RANTES, IP-10, MIP-1β, MIP-1α, and I-309. MDA-231 cells did not express mRNA for these chemokines within the assessed time period (24, 48 and 72 hours) at steady state or following irradiation (data not shown). Five other human breast cancer cell lines were also assessed for chemokine expression, all cell lines tested expressed mRNA for MCP-1 and IL-8 but not RANTES, IP-10, MIP-1β, MIP-1α, and I-309 (data not shown).
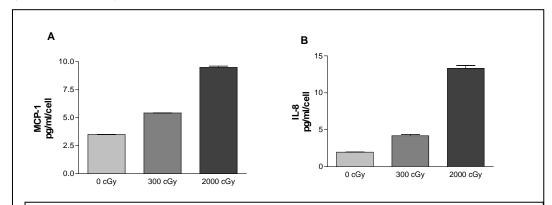

Figure 1. Irradiation increases production of the pro-angiogenic chemokines MCP-1 and IL-8 in MDA-231 breast cancer cells. MDA-231 was subjected to 0, 300, or 2000 cGy of gamma irradiation and then cultured at a density of 500,00 cells/ml. Supernatant was collected and analyzed 72 hours later for (A) MCP-1and (B) IL-8. To assess the amount of angiogenic chemokines produced on a per cell basis, the values obtained in the ELISA were divided by the number of cells per culture.

Additional "stressors" were assessed to confirm the reproducibility of the observation that production of pro-angiogenic chemokines are upregulated following growth arrest by gamma irradiation. Similar observations were observed with the combination of serum starvation under hypoxic conditions (figure 3). Serum starvation and hypoxia reduced the proliferation of MDA-231 but had no effect on viability at 24, 48 or 72 hours (data not shown). Both MCP-1 and IL-8 protein production per MDA-231 breast cancer cell was increased in treated cells following 72 hours in culture.

To assess if the production of IL-8 is tissue or cell line specific, the cell line ACHN, a renal cell carcinoma, which is known to produce constitutive IL-8 was stressed for 72 hours in the absence of fetal bovine serum. Under these experimental conditions proliferation was decreased but no effect on culture viability at 24, 48 or 72 hours was observed (data not shown). IL-8 production per cell was increased more than 2 fold after 72 hours (data not shown). Taken together, these results demonstrate that epithelial cancer cells can upregulate pro-angiogenic chemokines in response to "stress" conditions. It can be postulated that is response is a defense mechanism to enhance angiogenesis following injury or inadequate microenvironments.
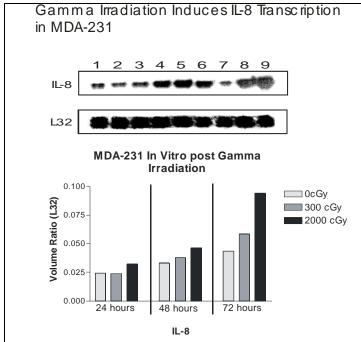
Gamma Irradiation Induces IL-8 Transcription in MDA-231

MDA-231 In Vitro post Gamma Irradiation

Figure 2: Effect of gamma irradiation on mRNA expression of IL-8. MDA-231 cells were exposed to 0 (lanes 1, 4, 7), 300 (lanes 2, 5, 8), or 2000 (lanes 3, 6, 9) cGy gamma irradiation and cultured for 24 (lanes 1, 2, 3), 48 (lanes 4, 5, 6), and 72 (lanes 7, 8, 9) hours. Total RNA was collected at the various time points and mRNA expression was determined by Ribonuclease Protection Assay. IL-8 bands were normalized to L32 levels.
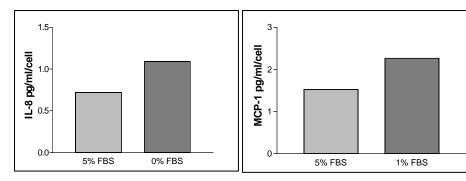
Figure 3: Serum deprivation induces increased production of IL-8 and MCP-1. MDA-231 cells were placed in serum deplete media in an incubator at 1% O_2 conditions for 72 hours. Supernatants were collected and analyzed for IL-8 and MCP-1 by ELISA. Results are calculated on a per cell basis.

Task 1b. To determine the effects of cytoreductive conditioning (i.e. chemotherapy or irradiation) on chemokine production by human BC lines placed in immunodeficient SCID mice. To determine if production of MCP-1 and IL-8 by MDA-231 was increased in response to irradiation or chemotherapy in vivo, subcutaneous tumors were established in SCID mice. When the tumors were an average of 5x5mm, the mice received 300 cGy whole body irradiation or a single dose of cytoxan (200 mg/kg). Induction of MCP-1 and IL-8 was greater with a single high

dose of cytoxan than low dose irradiation (Figure 4). The results were confirmed by measurement of serum human IL-8 in these mice (data not shown). The kinetics of chemokine production in response to a single high dose of cytoxan or daily low dose cytoxan was markedly different. While induction of both MCP-1 and IL-8 were rapid but quickly dropped off following high dose cytoxan the production of the chemokines in response to daily low dose (15 mg/kg) cytoxan was slower but increased by day 4 following initiation of treatment.
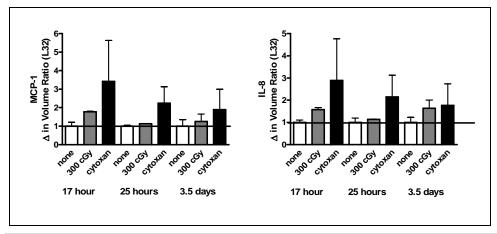

Figure 4. Administration of whole body irradiation or cytoxan to tumor bearing mice results in increased production of MCP-1 and IL-8 in tumor cells. One million MDA-231 breast cancer cells suspended in matrigel were injected into the mammary fat pad of CB.17 SCID mice. When the average tumor measurement reached 5 x 5 mm the mice were treated with 300 cGy whole body irradiation or a single dose of 200 mg/kg cytoxan. Mice were bled and tumors excised at 17, 25 and 84 hours following treatment.

Task 2. To determine the efficacy of chemokine blockade on human BC growth/progression. To address task 2, we assessed the effects of monoclonal antibodies to human IL8 and human MCP-1 on human BC growth in vitro under confluence and under "stress" conditions. As expected, neutralization of IL-8 and MCP-1 had no effect on viability of MDA-231 cells (Figure 5A). A slight and insignificant decrease in proliferation was observed when both chemokines were neutralized (Figure 5B). In comparison, proteasome inhibition with Velcade induced apoptosis and loss of MDA-231 cells (positive control, figure 5A and 5B). Overall, these data demonstrate that IL-8 and MCP-1 do not provide an autocrine signal for cell growth or survival in MDA-231 breast cancer cells. However, we have shown that neutralization of MCP-1 can significantly inhibit metastatic cell growth of MDA-231 in SCID mice resulting in prolonged survival of tumor bearing animals [1]. We have also previously shown that neutralization of anti-IL-8 does not enhance survival of MDA-231 tumor bearing mice but it can synergize with blockade of the EGF receptor to inhibit metastatic growth [2]. Therefore, studies were undertaken to determine if the combination of neutralization of MCP-1 and IL-8 synergize to inhibit metastatic cell growth.
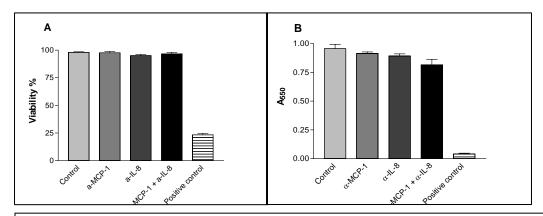
Figure 5: A. Anti-MCP-1 and anti-IL-8 alone or in combination had no effect on viability of MDA-231 at 72 hours. B. Anti-MCP-1 and anti-IL-8 alone or in combination did not effect the proliferation of MDA-231 in a MTT assay.

As shown above, the tumor line MDA-231 constitutively produces IL-8 and MCP-1, and that the production of these factors can be increased under stress conditions such as serum starvation or irradiation. To examine the in vivo effects of combined administration of blocking antibodies to these chemokines, we compared development of gross lung metastases 49 days after i.v. tumor inoculation. Blocking antibodies were administered at 25 ug/mouse day 4 through 28 every 4 days. As seen in Figure 6, treatment with anti-MCP-1 alone had a slight effect on lung metastasis, though this was not statistically significant. Treatment with anti-IL-8 showed a significant reduction in visible lung metastases versus both mouse IgG control and anti-MCP-1. No additional benefit was observed when anti-IL-8 was combined with anti-MCP-1 treatment.
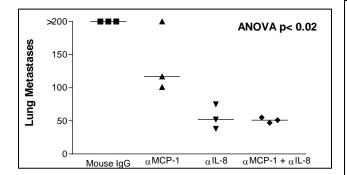

Figure 6. Anti-IL-8 treatment decreases the number of human BRCA lung metastases in a xenograft model. Mice were depleted of NK cells by administration of anti-AsGM1, they then received 50,000 MDA-231 cells iv. Anti-IL-8 (25 ug/dose) and/or anti-MCP-1 (25 ug/dose) was administered once every 4 days from day 4 to 28 post-tumor cell infusion. 3 mice per group were euthanized on day 49 post tumor injection, and whole lungs were assessed for the number of visible tumors on the surface of each organ. Metastases that were too numerous to count are expressed as >200.

In these same studies, we also examined serum chemokine levels in the mice at day 49 post tumor cell infusion. Serum MCP-1 was below detectable limits (31.5 pg/ml) in samples from all treatment groups (data not shown). Serum IL-8 was present in MDA-231 bearing mice that received isotype control antibody or anti-MCP-1 (Figure 7). However, in the animals that received anti-IL-8, serum IL-8 was significantly reduced or not detectable 25 days after the last injection of antibody. While we cannot rule out the continued presence of anti-IL-8 it is more likely that the low IL-8 levels in the serum was related to the reduction in the number of

metastatic lesions and therefore less tumor producing the IL-8. However, if this is true, the emergence of IL-8 negative variants will negatively impact the outcome of anti-IL-8 as a single agent. Additional studies will explore this potential response of the tumor to treatment and strategies to eliminate non-responders to anti-IL-8 treatment.
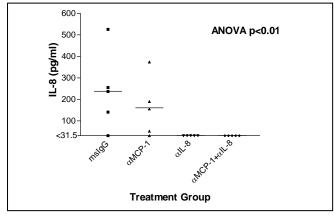

Figure 7. Anti-IL-8 results in decreased levels of serum IL-8 in MDA-231 tumor bearing mice. SCID mice were treated as described in Figure 1. Serum was obtained from 5 mice per group on day 49 post-MDA-231 cell infusion. IL-8 levels were determined by ELISA.

To further examine the effect of in vivo treatment with anti-IL-8 on tumor expression of chemokines we implanted subcutaneous tumor cells of MDA-231 into the flank of CB.17 SCID mice. Mice were then treated with anti-IL-8 or control antibody every 4 days from day 4 to 28 post-implantation. At day 49 post-implantation, tumors were excised and mRNA production in the tumors was assessed by RPA. In these experiments, we observed an increase expression of both IL-8 and MCP-1 mRNA in tumors from mice treated with anti-IL-8 (Figure 8). Rantes, MIP-1β, and MIP-1α were not detectable in any of the tumor samples. While additional experiments are needed to confirm the translation of IL-8 mRNA into protein, these data would suggest that 1) the tumor compensates for the neutralization of anti-IL-8 by increasing production of IL-8 and MCP-1 and 2) reduction of serum IL-8 following anti-IL-8 may be due to reduction in tumor burden and not emergence of IL-8 tumor variants. However, additional experiments are necessary to confirm this conclusion.
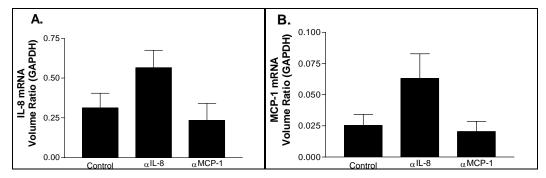

Figure 8. Anti-IL-8 increases the transcription of both IL-8 and MCP-1 genes. CB.17 SCID mice were received 2 million MDA-231 cells in 0.2 ml matrigel s.c. Mice then received anti-IL-8 (25 ug/dose), anti-MCP (25 ug/dose) or mouse IgG (25 ug/dose) was administered once every 4 days from day 4 to 28 post-tumor implantation. Tumors were harvested on day 49 post-implantation. Total RNA was extracted from individual samples and IL-8 and MCP-1 transcription levels were determined by RNAse protection assay. Densitometric analysis was performed and is shown as a ratio of the sample volumes normalized to the expressed volumes of the housekeeping gene, GAPDH.

To explore the mechanism by which neutralization of anti-IL-8 reduced lung metastases growth in the MDA-231 xenograft model, we reexamined our data that demonstrated that anti-IL-8 did not directly affect MDA-231 proliferation in vitro. In these new experiments, we increased the concentration of anti-IL-8 used to treat MDA-231. Cells were cultured for 72 hours and examined the effect of various concentrations of anti-IL-8 on proliferation was determined (Figure 9). We were able to show that the growth of MDA-231 is inhibited in a dose dependent manner with high concentrations of anti-IL-8. Therefore, anti-IL-8 has direct inhibitory effects on the growth of this breast cancer cell line. In addition, we have previous shown that anti-IL8 can block endothelial cell migration and therefore block tumor escape via angiogenesis[2]. In contrast to treatment with anti-IL-8 in vitro, a 72 hours treatment with anti-MCP1 had no effect on proliferation (data not shown), implying that the effects on lung metastases observed in-vivo are most likely indirect. This is consistent with our previous results which showed that MCP-1 plays a role in the migration of endothelial cells in the tumor microenvironment, but does not act directly on the tumor[1].
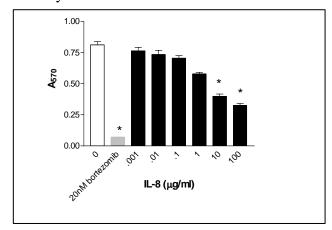

Figure 9. Anti-IL-8 decreases the proliferation of MDA-231 in vitro. MDA-231 cells (2000 cells/well in triplicate) were plated in 96 well plates with anti-IL-8(0-100 μg/ml) and cultured for 72 hours and then analyzed by the MTT assay. The results are representative of two experiments. Significant differences were determined by ANOVA (*P<0.001, compared to untreated group).

Task 3. To determine the effects of chemokine blockade in conjunction with chemotherapy/irradiation. To initiate these studies, we examined the effect of irradiation on chemokine production in tumors that had been treated with chemokine blockade in mice followed by sublethal irradiation. Mice were implanted with s.c. MDA-231 cells and treated with anti-IL-8, anti-MCP-1 or control antibody as described in Figure 8. In this experiment the mice then received a sublethal dose of total body irradiation (300 cGy). Chemokine expression in the tumors was determined 2 days later. As seen in Figure 10, tumors from mice treated with chemokine blockade prior to sublethal irradiation produced more mRNA for IL-8 and MCP-1 than mice that received irradiation or chemokine blockade alone. Rantes, MIP-1β, and MIP-1α transcripts were not detectable in any of the tumor samples. These results demonstrate the ability of the tumors cells to respond to the treatment by increasing the production of chemokines. Therefore, higher dosing of neutralizing chemokine antibodies may be needed for therapeutic effects in tumor-bearing animals following treatment with cytoreductive conditioning.
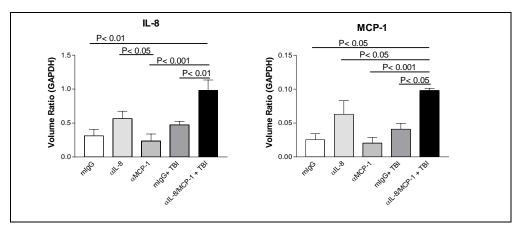
Figure 10. Compensatory increase in chemokine expression by MDA-231 is additive following anti-IL-8 and sublethal total body irradiation (TBI). Subcutaneous tumors were established in CB17 SCID mice as described in figure 8. Mice then received anti-IL-8 (25 ug/dose), anti-MCP (25 ug/dose) or mouse IgG (25 ug/dose) was administered once every 4 days from day 4 to 28 post-tumor implantation. Mice received 300 cGy TBI when the tumors reached 5x5mm (day 59). Tumors were harvested on day 61 post-implantation. Total RNA was extracted from individual samples and IL-8 and MCP-1 transcription levels were determined by RNAse protection assay. Densitometric analysis was performed and is shown as a ratio of the sample volumes normalized to the expressed volumes of the housekeeping gene, GAPDH.

In the last half of the third year, studies to determine if chemokine blockade following cytoxan treatment in a metastatic tumor model were attempted. However, no the attempted protocols did not result in improvement in overall survival. Follow-up studies will be required to determine if the optimal dose and schedule were employed in these studies.

KEY RESEARCH ACCOMPLISHMENTS:

- Anti-IL-8 and anti-MCP-1 can inhibit the formation of human breast cancer lung metastasis in a xenograft model.
- MDA-231 cells increase there production of IL-8 and MCP-1 in vivo in response to stress including cytoreductive conditioning and neutralization of IL-8.
- IL-8 but not MCP-1 appears to function as autocrine growth or survival factor for MDA-231 cells in vitro. High concentrations of neutralizing antibody are required to observe the effect.

REPORTABLE OUTCOMES: Manuscript in preparation

CONCLUSIONS:

Chemokines have been shown to provide angiogenic effects in cancer. Blockade of chemokines can produce anti-tumor effects on metastatic growth of human breast cancer cells in a xenograft model. However, the effects of "stress", whether in the form of radiation or chemotherapy, may enhance the tumor cells attempts to promote angiogenesis via increased production of

chemokines and thus be more susceptible to chemokine blockade. We have demonstrated that MDA-231 breast cancer cells produce IL-8 and MCP-1 but not RANTES or MIP-1. We have previously shown that both mRNA and protein levels of MCP-1 and IL-8 are increased following "stress" of the tumor cells by gamma irradiation or serum starvation and hypoxia. In this report we demonstrate that IL-8 and MCP-1 transcripts are increased in vivo in response to IL-8 neutralization and/or gamma irradiation. In contrast, serum concentration of IL-8 was decreased in response to in vivo treatment with anti-IL-8, 25 days following cessation of treatment. This apparent discrepancy between the mRNA and protein most likely is due to an increase in IL-8 production per tumor cell but overall reduction in the number of tumor cells in vivo. We have also demonstrated that IL-8 but not MCP-1 can function as autocrine growth or survival factor for MDA-231 cells in vitro but neutralization requires high concentrations of antibody.

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